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Comparative Evaluation of Molecular Detection Performance of *Pseudomonas aeruginosa* based on Phylogenetic Markers 16S RNAr, *rec*A, *rpo*B and ITS1

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Abstract

P. aeruginosa may be involved in the poisoning of food. It is highly pathogenic to immunocompromised subjects or weakened, causing a high rate of morbidity and mortality. The aim of this study was to determine the phylogenetic marker suitable for molecular identification of *Pseudomonas aeruginosa*. The purity and concentration of the nucleic acids were determined by spectrophotometry. Sensitivity reactions using phylogenetic markers (16S RNAr, *rec*A, *rpo*B, STS1) and the threshold detection of 42 strains were assessed by polymerase chain reaction (PCR). With an average absorption at 230 nm of 2.1, the DNA extracts has an average ratio (A260/A280) of 1.7. The threshold detection of *Pseudomonas aeruginosa* reference strain ATCC 27853 was 0.8 µg/ml for *rpo*B and 7.6 µg/ml for each of 16S markers RNAr and *rec*A. The threshold detection of positive control strains CP_2: 1125A and CP_3: API was 1.2 µg/ml and 0.1 µg/ml for using *rpo*B gene, respectively. This threshold was respectively 12.3 µg/ml and 0.9 µg/ml for the *rec*A gene. The sensitivity of the *rpo*B housekeeping gene was 97.4% followed by the *rec*A and 16S RNAr with 87.2% and 82.1%, respectively. The phylogenetic resolution of the *rpo*B genes was higher than that of the 16S rRNA and *rec*A genes. No sensitivity reaction was observed with ITS1 marker. The quality, purity of the nucleic acids and the choice of phylogenetic marker are among the most critical factors for PCR analysis.

Keywords: *Pseudomonas aeruginosa*; Phylogenetic marker; PCR; Sensitivity

Introduction

Pseudomonas aeruginosa is a ubiquitous bacterium found in the environment such as soil, water, inert surfaces, plants, antiseptic solutions and foods [1-3]. It is also a commensal of digestive tract that is scarce in the healthy human (2-10% of carriers), but the proportion in immunocompromised persons may reach 50% or even 60% of burn wounds in some areas [4]. The bacteria can be in planktonic form, sessile condition or in a biofilm [5,6].

Pseudomonas aeruginosa is an opportunistic pathogen classified principally third major agent of nosocomial infections after *Escherichia coli* and *Staphylococcus aureus* [7]. The pathogenicity of species is attributed to the production of several virulence factors and an emergence of new antibiotic resistance genes [8-10]. Infections caused by multiresistant strains of *Pseudomonas* are a major health problem [11].

Serotyping and molecular characterization of strains, play an important role in the management of infections associated with *Pseudomonas aeruginosa* [12-14]. Phylogenetic characterization of the strains most often requires using of certain household markers or phylogenetic markers. These include RNAr 16S (ribosomal RNA), *rec*A (recombinase A) *rpo*D (Ϭ70 factor RNA polymerase), *gyr*B (in β unit of DNA gyrase), *rpo*B (subunit β RNA polymerase) and ITS1 (spaceur intergenic transcribed) ("Internal transcribed Spacer") region between 16S-23S rDNA allow the differentiation of *Pseudomonas* species [9,15]. The variability of the results obtained by using each marker and one author to another [9,14,16], show a necessity for standardization of phylogenetic characterization methods.

Strains of *Pseudomonas aeruginosa* isolated from our environment (in hospitals) were identified by classical bacteriological techniques, based on the determination of phenotypic traits [9,15,17]. Not only molecular detection of strains has previously been carried out, but also some unknown marker is suitable for phylogenetic characterization and molecular identification of strains.

The objective of this study was to determine the proper phylogenetic marker for molecular identification of *Pseudomonas aeruginosa* strains.

Material and Methods

Pseudomonas aeruginosa strains

Forty-two (42) strains of *Pseudomonas aeruginosa*, consisting of thirty-nine (39) strains isolated from animal products and three (3) positive control strains from Institut Pasteur of Cote d'Ivoire were used in this study. The 3 positive controls are *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* 1125A and *Pseudomonas aeruginosa* API.

DNA extraction

In total, three (3) positive controls and thirty nine (39) presumptive isolates of *Pseudomonas aeruginosa* were used. Genomic DNA was extracted by boiling and purified according to the method previously described [18]. After culturing for 24 hours in 2 ml Brain Heart broth (Biokar Diagnostics, BK015HA, France) at 37°C, the strains were grown on Müeller Hinton agar (Biokar Diagnostics, BK048HA, France) for 18-24 h. Three (3) colonies were picked and suspended in 1 ml of Milli-Q water (milli-Q™, Millipore Corporation, USA) sterile. A volume of 200 ml of each sample is removed and placed in sterile Eppendorf tubes labeled beforehand. The tubes are first incubated at 20°C for 15 minutes and then at 95°C for 15 minutes to create thermal shock.

The supernatant was collected in sterile Eppendorf tubes after centrifugation at 14000 rpm for 10 mins and stored at -20°C for PCR. The DNA is purified with 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1, EV Canada) and centrifuged at 1300 rpm for 10 min at 4°C. The aqueous upper phase is recovered and measured into a sterile Eppendorf tube. A 1/10ème of a volume of sodium acetate (ID: 3620574, USA) 3M and 500 µl of absolute ethanol (Agilent Technologies, CA, USA) stored at -20°C are added to the aqueous solution and incubated at -20°C for 18 h. The tubes are centrifuged at 1300 rpm for 20 min at +4°C and the supernatant is eliminated. A volume of 1 ml of 70% ethanol, stored at -20°C was added to the pellet and the tubes are centrifuged at 1300 rpm for 10 min at +4°C. After removing the supernatant, the pellet is dried on dry bain-marie at 70°C and re-suspended in 60 µl of sterile Milli-Q water (milli-Q™, Millipore Corporation, USA). The purity and concentration of DNA in the extract were determined with a spectrophotometer (Eppendorf BioPhotometer plus, USA).

Mixture reaction of gene amplification

After determining purity and concentration of DNA in the extract by spectrophotometry decimal dilutions ranging from 10^{-1} to 10^{-10} were conducted to the DNA suspension constitution for each strain. The different DNA suspension lines were used as template DNA in the mixture reaction to determine the threshold detection and sensitivity by PCR, targeting housekeeping genes RNAr16S, *rec*A, *rpo*B and ITS1.

All the PCR were performed in a volume of 25 µl of bacterial genomic DNA solution consisted of 16 µl of sterile Milli-Q water (milli-Q™, Millipore Corporation, USA), 5 µl of loading buffer (5X) concentration, 1.5 µl of $MgCl_2$ (2 mM) (Promega Corporation, Madison, WI 53711-5399, USA), 0.2 µl dNTPs (10 mM), 0.1 µl of each primer (20 mM) (Integral DNA Technology, France), 0.1 µl of Go Tag G_2 Flexi DNA polymerase final concentration 1.5 U (Promega Corporation, Madison, WI 53711-5399, USA) and 2 µl of the DNA template. Water sterile Milli-Q and the reference strains of *Pseudomonas aeruginosa* ATCC 27853 were used respectively as negative control and positive control for each PCR reaction.

Amplification of 16S rRNA, *rec*A, *rpo*B and ITS

The amplification of 16S rRNA gene for *Pseudomonas* detection was performed according to the method described. The determination of *Pseudomonas aeruginosa* is carried out by amplification of specific fragments of *rec*A, *rpo*B and STI1. The amplification of *rec*A, *rpo*B and ITS genes has achieved respectively by using primer pair *rec*-AS and *rec*A-AS; *rpo*B-F and *rpo*B-R and 16F945 and 23R458 as described [19-22]. The amplification program and the nucleotide sequence of the primers used are described in (Table 1). The amplification reactions were performed in a thermocycler type T3000 Thermocycler, Block standard type 3a (Biometra, Germany). 10 µl of PCR products were revealed on a 2% agarose gel for 30 min at 120 V. A volume of 5 µl of a molecular weight marker (Bench Top, 1kb DNA Ladder, Promega Corporation, USA) was included. The gels were prepared in 1X TAE buffer containing 2.5 mg/L of ethidium bromide solution and visualized by UV transilluminator (Molecular Imager Gel DocTM EZ, Bio-Rad, USA).

Target genes	Primers	Sequence of the Primers (5'-3')	Amplification program	Product size (bp)	Annealing temperatue (°C)	References
16S rADN	16S-F	AGAGTTTGATCCTGGCTCAG	94°C, 2 min 5x (94°C, 45 s; 55°C, 1min; 72°C, 2 min) 35 x (92°C, 45 s; 60°C, 45 s; 72°C, 2 min) 72°C, 2 min	≈1351	55	[22]
	16S-R	CTACGGCTACCTTGTTACGA			60	
recA	rec-AS	ATGGACGAGAACAAGAAGCG	94°C, 3 min; 30 x (94°C, 1min; 58°C, 1min; 72°C, 2 min); 72°C, 2 min; 4°C	1041	58	[20]
	recA-AS	TCAATCGGCTTCGGCGTC				
					58	
rpoB	rpoB-F	CAG TTC ATG GAC CAG A AC AAC CCG		759		[23]
	rpoB-R	ACGCTGGTTGATGCAGGTGTTC				
ITS	16F945	GGGCCCGCACAAGCGGTGG	94°C, 5 min; 30 x (94°C, 1 min; 55°C, 1 min; 72°C, 2 min); 72°C, 2 min	≈1300	55	[22]
	23R458	CTTTCCCTCACGGTAC				

Table 1: Nucleotide sequence of the primers used for PCR and the amplification program.

Sensitivity evaluation

The sensitivity of molecular test refers to the ability of the test to correctly identify *Pseudomonas aeruginosa* strains in samples with the phylogenetic marker. This test demonstrates the most sensitive and rapid method using phylogenetic markers for the detection of *Pseudomonas aeruginosa* strains. When evaluating a molecular test,

the expression for calculating sensitivity according to Ghaaliq and McCluskey is as follows [21].

$$Sensitivity = \frac{True\ Positives}{True\ Positives\ +\ False\ Negatives}$$

In that expression, True positive show that *Pseudomonas aeruginosa* is present in the sample and the test is positive; while, false positive show that *Pseudomonas aeruginosa* is not present in the sample but the test is positive. In this study, all strains of *Pseudomonas aeruginosa* studied were isolated, identified and stored in the laboratory with well-known characteristics. The various molecular tests allowed for selecting the appropriate phylogenetic marker to confirm this identification.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) Version 22; SPSS Inc., Chicago, IL, USA. The P value of <0.05 was deemed as statistically significant.

Results

Concentration and purity of the extracted nucleic acids

The DNA extracts has an average ratio (A260/A280) of 1.7. This ratio is close to 1.8 and indicates the purity of DNA preparations concerning proteins, phenol or agarose. Mean absorption at 230 nm is 2.1 and also reflects the non-contamination of the preparations by substances such as carbohydrates, peptides or aromatic compounds (Table 2).

No order	Sample Type	Purity A_{260}/A_{280}	Purity A_{260}/A_{230}	Concentration (µg/ml)
1	CP_1 : ATCC 27853	1.7	2.1	75.5
2	CP_2 : 1125A	1.7	2.2	122.7
3	CP_3 API	1.7	2.1	88.5

Table 2: Values of absorbance and concentration of DNA extracts from positive controls (CP: positive control).

Detection of *Pseudomonas aeruginosa* strains by phylogenetic marker

The detection limits were evaluated with the three positive controls strains from the genes of households 16S RNAr, *rec*A, *rpo*B and STI1. With an initial DNA concentration of 75.5 µg/ml and a purity of 1.7 for *Pseudomonas aeruginosa* ATCC 27853 reference strain, good resolution is achieved only with the *rpo*B gene for a detection limit to 0.8 µg/ml (Table 2 and 3). *Pseudomonas aeruginosa* ATCC 27853 reference strain threshold detection by each of 16S markers RNAr and *rec*A was 7.6 µg/ml (Figures 1-3). The best threshold detection of positive controls strains CP_2: 1125A and CP_3: API was respectively 1.2 µg/ml and 0.1 µg/ml with *rpo*B marker. The threshold detection with the *rec*A gene was 12.3 µg/ml for strain CP_2: 1125A (Figures 4-8) and 0.9 µg/ml for strain CP_3 API. These thresholds detection of strains CP_2: 1125A and CP_3: API has been obtained with DNA initial concentrations respectively of 122.7 µg/ml and 88.5 µg/ml for a purity of approximately 1.7 (Tables 2 and 3; Figure 9-12). No threshold detection was observed with ITS1 gene (Figure 4).

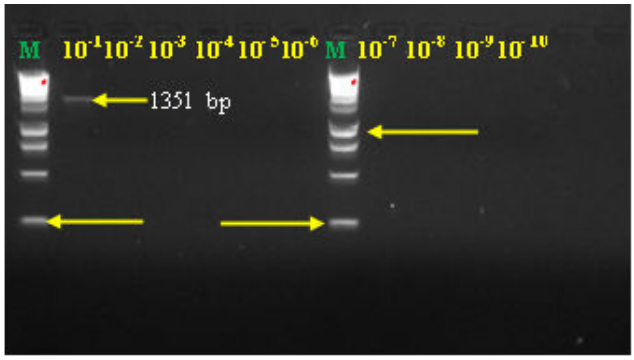

Figure 1: Electrophoretic profile showing the threshold detection of the 16S RNA gene from *P. aeruginosa* ATCC 27853 (M: Marker Gene Ruler 250 bp).

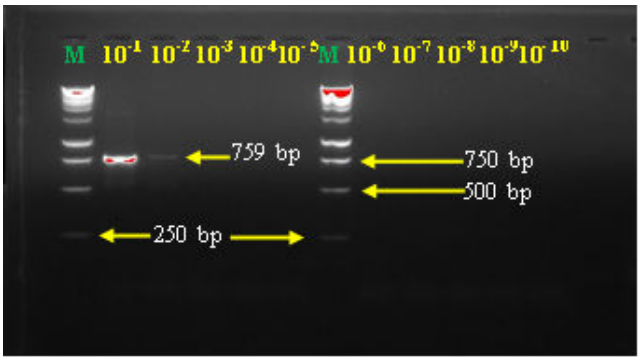

Figure 2: Electrophoretic profile showing the threshold detection of the *rpo*B gene from *P. aeruginosa* ATCC 27853(M: Marker Gene Ruler 250 bp).

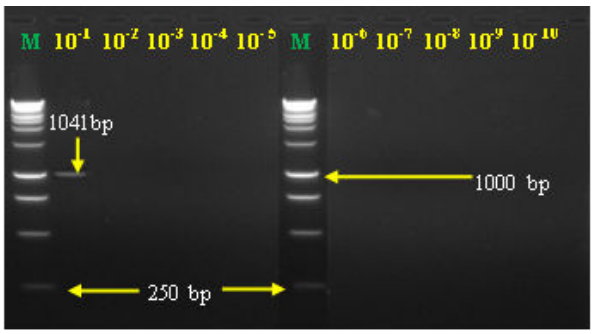

Figure 3: Electrophoretic profile showing the threshold detection of the *rec*A gene from *P. aeruginosa* ATCC 27853 (M: Marker Gene Ruler 250 bp).

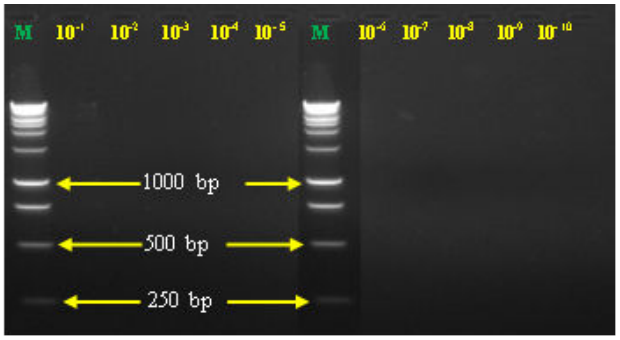

Figure 4: Electrophoretic profile showing the threshold detection of the ITS gene from *P. aeruginosa* ATCC 27853 (M: Marker Gene Ruler 250 bp).

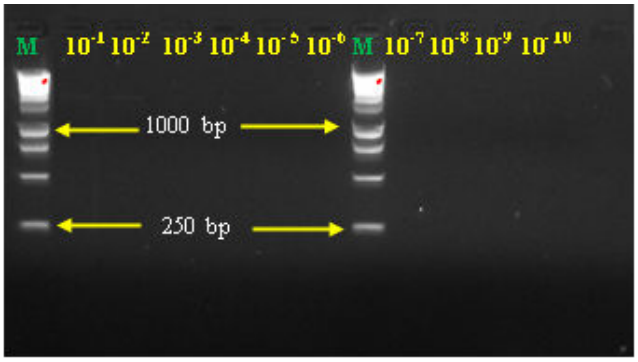

Figure 5: Electrophoretic profile showing the threshold detection of the 16S RNA gene from *P. aeruginosa* 1125A (M: Marker Gene Ruler 250 bp).

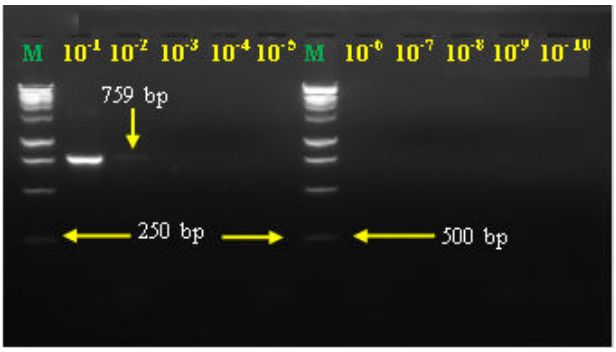

Figure 6: Electrophoretic profile showing the threshold detection of the *rpo*B gene from *P. aeruginosa* 1125A. (M: Marker Gene Ruler 250 bp).

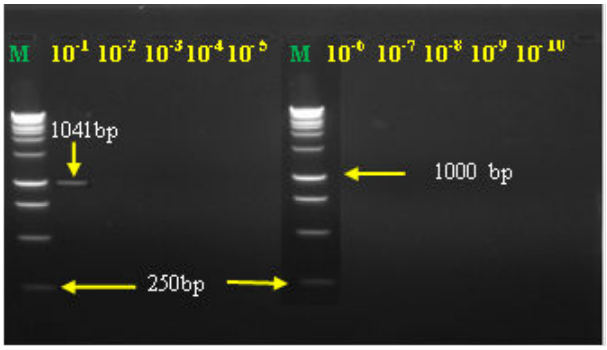

Figure 7: Electrophoretic profile showing the threshold detection of the *rec*A gene from *P. aeruginosa* 1125A.(M: Marker Gene Ruler 250 bp).

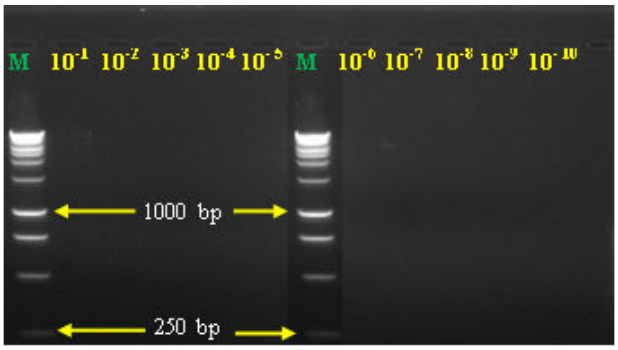

Figure 8: Electrophoretic profile showing the threshold detection of the ITS gene from *P. aeruginosa* 1125A (M: Marker Gene Ruler 250 bp).

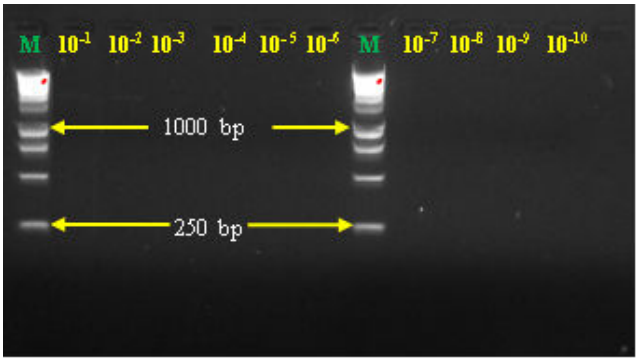

Figure 9: Electrophoretic profile showing the threshold detection of the 16S RNA gene from *P. aeruginosa* API (M: Marker Gene Ruler 250 bp).

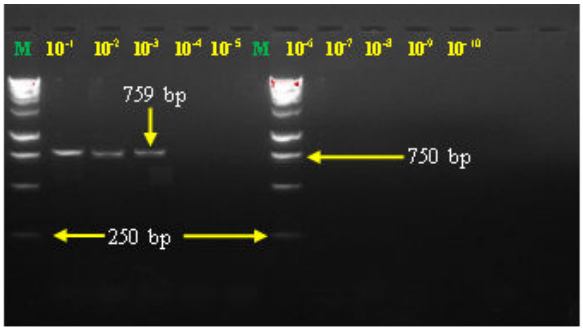

Figure 10: Electrophoretic profile showing the threshold detection of the *rpo*B gene from *P. aeruginosa* API (M: Marker Gene Ruler 250 bp).

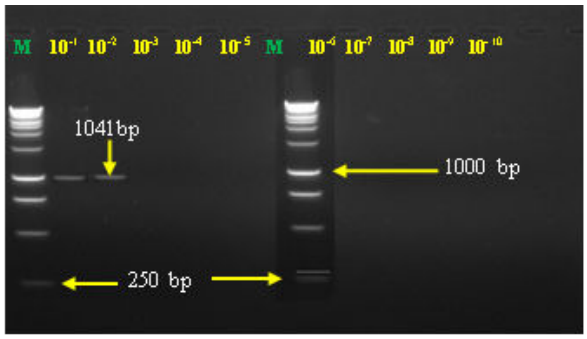

Figure 11: Electrophoretic profile showing the threshold detection of the *rec*A gene from *P. aeruginosa* API (M: Marker Gene Ruler 250 bp).

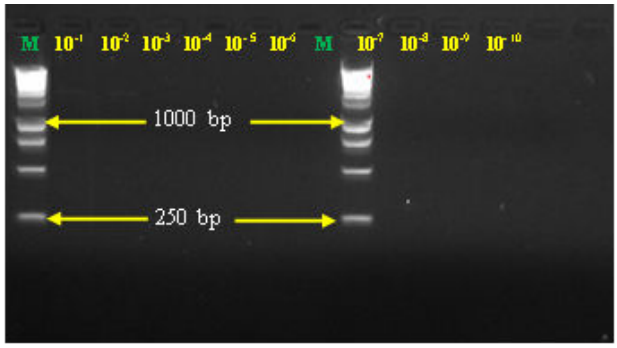

Figure 12: Electrophoretic profile showing the threshold detection of the ITS gene from *P. aeruginosa* API.(M: Marker Gene Ruler 250 bp).

Sensitivity of different phylogenetic markers for stem tests

The sensitivity of the reaction with using four markers phylogenetic 16S RNAr, *rec*A, *rpo*B and ITS1 for *Pseudomonas aeruginosa* identification indicates that a sensitivity of the *rpo*B housekeeping gene is 97.4% followed by the *rec*A and 16S RNAr respectively 87.2% and 82.1% (Table 4) (Figures 13 to 15). However, no sensitivity reaction was observed with using ITS1 marker (Table 4 and Figure 16).

	Initial concentration of positive control DNA (µg/ml)		
Phylogenetic marker	CP_1: ATCC 27853	CP_2: 1125A	CP_3: API
	75.5	122.7	88.5
	Threshold detection		
16S RNAr	7.6_a	-	-
*rpo*B	0.8_b	1.2_a	0.1_a
*rec*A	7.6_a	12.3_b	0.9_b
ITS1	-	-	-

Table 3: Threshold detection of *Pseudomonas aeruginosa* strains by phylogenetic markers. Each index letter indicates threshold detection values for the various phylogenetic markers whose column proportions do not differ significantly from each other in level 05. The Threshold detection indicates the lowest concentration of DNA from which each phylogenetic marker can always detect the strains of *Pseudomonas aeruginosa* with a known initial concentration (CP: positive control).

Phylogenetic Markers	Sensitivity and phylogenetic markers for detecting Pseudomonas aeruginosa strains in animal product			Total number of samples tested
	True Positives	False Negatives	Sensitivity (%)	
RNA16S	32_a	7_a	82.1	39
*rpo*B	38_a	1_a	97.4	39
*rec*A	34_a	5_a	87.2	39
ITS1	0.0_b	39_b	0	39

Table 4: Sensitivity of the detection reaction by using phylogenetic markers for testing *Pseudomonas aeruginosa* strains in animal product. Each index letter indicates a subset of gene categories whose column proportions do not differ significantly from each other in level 05.

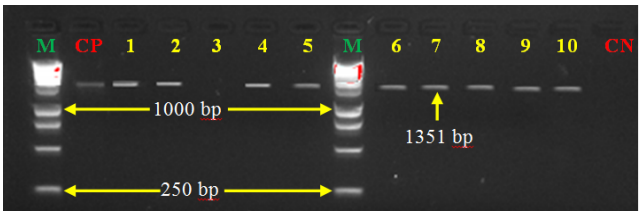

Figure 13: 16S rRNA electrophoretic profiles of *Pseudomonas aeruginosa* identification. Lanes 1-2, 4-10: Positives samples; Lane 3: Negatives samples (M: Marker Gene Ruler 250 bp; CP: Positive Control; CN: Negative Control).

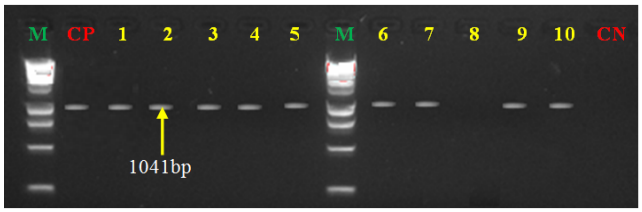

Figure 14: *Rec*A electrophoretic profiles of *Pseudomonas aeruginosa* identification. Lanes1-7, 9-10: Positives samples; Lane 8: Negatives samples (M: Marker Gene Ruler 250 bp; CP: Positive Control; CN: Negative Control).

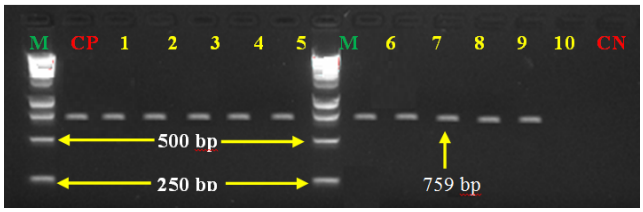

Figure 15: *Rpo*B electrophoretic profiles of *Pseudomonas aeruginosa* identification. Lanes 1-10: Positives samples (M: Marker Gene Ruler 250 bp; CP: Positive Control; CN: Negative Control).

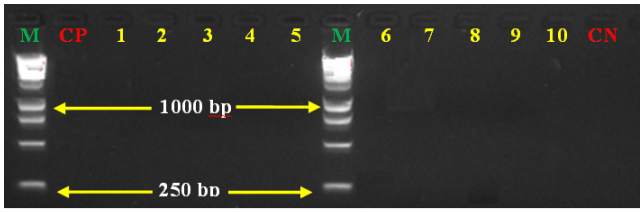

Figure 16: ITS1 electrophoretic profiles of *Pseudomonas aeruginosa* identification. Lanes 1-10: Negatives samples (M: Marker Gene Ruler 250 bp; CP: Positive Control; CN: Negative Control).

Discussion

Pseudomonas aeruginosa is a nosocomial pathogen responsible for morbidity and mortality in immunocompromised patients [10,24,25]. Laboratory diagnosis of infections caused by this bacterium is most commonly achieved by conventional methods such as growth in specific culture media [26,27].

In this study, molecular identification by using gene 16S RNAr households, *rec*A, *rpo*B and STI evaluated the detection threshold and the sensitivity of the reaction. All DNA extracts has a ratio A260/A280 medium of 1.7. This ratio is close to 1.8 and indicates the purity of all DNA preparations concerning proteins, phenol or agarose [28].

The purity obtained, could be justified by the quality or the conditions of the extraction method. Therefore, these purified nucleic acids could allow to carry out a specific analysis of *Pseudomonas aeruginosa* detection using the polymerase chain reaction (PCR). The quality and purity of nucleic acids are among the most critical factors for PCR analysis by Urakawa [29]. The reference strain detection level *Pseudomonas aeruginosa* ATCC 27853 from the genes of households showed that with the *rpo*B gene, good resolution is obtained for a detection limit of 0.8 µg/ml. The best resolution of the *rpo*B gene could be due to the performance of the reaction with the species *Pseudomonas aeruginosa* [9,30]. The detection reaction by using 16S RNAr and *rec*A has presented the same threshold of detection which is 7.6 µg/ml. This result could be explained by the fact that the RNAr 16S enables the identification of the genus Pseudomonas; while the *rec*A gene allows identifies the species *Pseudomonas aeruginosa* and is most often associated with the 16S RNAr for complete identification [9,20,30].

For the positive controls CP_2: 1125A and CP_3: API, using of *rpo*B gene was also allow to get the best threshold of detection respectively 1.23 µg/ml and 0.1 µg/ml followed by *rec*A 12.3 µg/ml and 0.9 µg/ml. For the positive controls CP_2 : 1125A and CP_3: API, the using of *rpo*B gene was also allow to get the best threshold of detection respectively 1.2 µg/ml and 0.1 µg/ml followed by *rec*A 12.3 µg/ml and 0.9 µg/ml. The results obtained with the RNAr 16S gene could be explained by the fact that 16S gene RNAr is the key molecule which is based on the classification of prokaryotes, including that of *Pseudomonas* [31].

Unlike to the study performed by Franzetti and Scarpellini [22], no threshold detection was observed with the ITS1 gene. This result discrepancy could be explained by the sensitivity and performance of the method used. Indeed, in order to increase the number of comparable characteristics, other authors have found that the ITS-PCR typing can be improved by digestion of the PCR products with the restriction endonucleases [32].

The study also tested the sensitivity of the three markers phylogenetic 16S RNAr, *rec*A and *rpo*B for the identification of *Pseudomonas aeruginosa*. The sensitivity of the reaction with the housekeeping gene *rpo*B was 97.4% followed by the *rec*A and 16S RNAr with 87.2% and 82.1% respectively. The high sensitivity by using *rpo*B gene could be justified by the fact that the functional genes such as *rpo*B could be used to target a more precise phylogeny and for a higher taxonomic resolution, because of its essential role in cellular metabolism [33]. This high positivity rate with *rpo*B gene also indicates that *rpo*B gene can be the best identifier tag of *Pseudomonas aeruginosa* species. These same results were obtained by Tayeb et al. [33] which stated in their study that the gene *rpo*B resolution gave a three (3) times greater than that obtained with the 16S rRNA gene.

However, ITS1 housekeeping gene showed no sensitivity to the presumptive identification of *Pseudomonas aeruginosa* strains. The study also showed that all test animal strains amplified with 16S rRNA genes (≈1351 pb), *rec*A (1041 pb) and *rpo*B (≈759) were the same size as *Pseudomonas aeruginosa* ATCC 27853 reference strain and this confirms the membership of these animal strains to *Pseudomonas aeruginosa* species. Data from this study indicate that phylogenetic markers: 16S rRNA, *rec*A and mainly *rpo*B used can be exploited for confirmation of *Pseudomonas aeruginosa* strains of different origin.

Conclusion

Data from this study indicate that phylogenetic markers: 16S rRNA, *rec*A and *rpo*B used can be exploited for *Pseudomonas aeruginosa* strains confirmation of different origin. The study also showed that the sensitivity of the reactions was higher by using *rpo*B gene. This phylogenetic marker can therefore be recommended for the molecular identification of the *Pseudomonas aeruginosa* species.

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